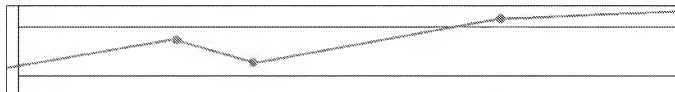





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doi:10.1016/S0264-410X(01)00364-4

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Preclinical evaluation of group B streptococcal polysaccharide conjugate vaccines prepared with a modified diphtheria toxin and a recombinant duck hepatitis B core antigen^a

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Received 31 May 2001; revised 25 July 2001;

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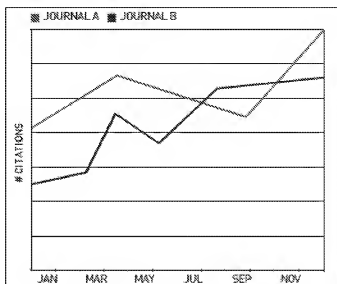
Abstract

An effective vaccine against group B streptococcal (GBS) disease will undoubtedly include capsular polysaccharides (CPSs) from each of the five serotypes prevalent in the United States individually coupled to immunogenic proteins. This formulation may require the use of two or more different protein carriers. We preclinically examined the potential of two proteins to serve as effective carriers for GBS type III CPS. Recombinant duck hepatitis B core antigen (rdHBcAg), a particulate protein of viral origin, and a newly mutated form of diphtheria toxin (DTm) were covalently and directly coupled to purified type III CPS by reductive amination. Seventy-seven of 79 (97%) newborn pups born to mouse dams actively vaccinated with type III CPS-rdHBcAg conjugate survived GBS type III challenge, whereas none of the pups born to dams that received an uncoupled mixture of type III CPS and rdHBcAg or saline survived. Likewise, 64 (98%) of 65 pups born to dams vaccinated with type III CPS-DTm conjugate survived challenge, in sharp contrast to no survivors among the pups born to dams vaccinated with an uncoupled mixture of type III CPS and DTm. The presence of type III CPS-specific IgG in serum from dams correlated with pup survival in groups that received a conjugate vaccine, and this serum was opsonically active *in vitro* against GBS type III. In addition, carrier-specific IgG was also measured in serum from vaccinated mice. These data suggest that the rdHBcAg and DTm may be effective carriers for GBS CPSs.

Author Keywords: Carrier proteins; Particulate antigens; Duck hepatitis B core antigen; Diphtheria protein; Maternal vaccination; Neonatal infections; Glycoconjugates; Women's health; Perinatal infections; Group B streptococci

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1. Introduction

In conjugate vaccines, carrier proteins covalently coupled to bacterial capsular polysaccharide (CPS) serve mainly to provide T cell-dependent help for the T cell-independent carbohydrate. In some cases, an immunogenic protein isolated from the same pathogen as the CPS can broaden vaccine coverage. For example, the alpha C protein of group B streptococcal (GBS) not only provided T cell help to the coupled type III CPS, but also induced functionally active antibody to non-type III GBS that possessed the alpha C protein [1]. Alternatively, carrier proteins can simultaneously induce functional antibody against a pathogen different from that targeted by the CPS antigen.

Preclinical GBS conjugate vaccines have been prepared using not only tetanus toxoid (TT), but also GBS alpha and beta C proteins [2 and 3] and streptococcal C5a peptidase [4]. Clinical lots of GBS conjugate vaccines have almost exclusively used TT as the carrier because this protein has been administered safely worldwide to pregnant women [5]. Immunization with an effective GBS vaccine during pregnancy is a strategy proposed to prevent neonatal

GBS disease [6].

Because of the concerns of the overuse of TT in vaccines, we sought to explore the use of other proteins as carriers for GBS CPS, specifically a particulate protein of viral origin (recombinant duck hepatitis B core antigen, rdHBcAg) and a recently derived mutant diphtheria toxin (DTm), a soluble protein of bacterial origin.

Since the presence of hepatitis B core-specific antibody is widely used as a method of screening hepatitis B virus (HBV)-positive individuals from the blood donor pool, the widespread use of HBcAg as a carrier protein would compromise such screening procedures. Duck hepatitis B virus is a hepadnavirus related to HBV that directs the synthesis of a core antigen which is physically quite similar to HBcAg; however, these two antigens do not cross-react immunologically (Peterson, unpublished results). Immunization of mice with rdHBcAg elicits specific antibody with titers equal to those of HBcAg. Therefore, we anticipated that rdHBcAg would be a good carrier protein for carbohydrate antigens, without the drawback of inducing HBcAg-specific antibody, and preexisting HBcAg-specific antibody would not be expected to influence the immunogenicity of vaccines containing rdHBcAg.

Cross-reactive material 197 (CRM₁₉₇), a mutant toxin enzymatically attenuated form of diphtheria toxin (DT) containing a mutation of glycine-52 to glutamic acid, has been commonly employed as a carrier protein in constructing CPS conjugate vaccines [7] including GBS type V CPS [8]. As an alternative to CRM₁₉₇, we tested another mutated form of DT, which contained two charge-reversal mutations: K51E and E148K (DT-51E148K) [9] to function as a carrier protein. This combination of mutations was identified in selection protocols in yeast designed to yield mutant forms of the A-chain of DT in which enzymatic activity was completely ablated (a million-fold lower activity than wild-type). The whole toxin containing the K51E and E148K mutations (DTm) is stable and entirely nontoxic, and it can be produced in *Escherichia coli* in high yields.

In separate reactions, rdHBcAg and DTm were covalently coupled directly to GBS type III CPS by

reductive amination [10]. The resulting conjugate vaccines were evaluated for immunogenicity and efficacy in a mouse maternal immunization–neonatal pup challenge model of GBS disease. Conjugate vaccine-induced antiserum was tested for in vitro functional activity against GBS and for the presence of carrier-specific antibody.

2. Materials and methods

2.1. Preparation of GBS type III polysaccharide–diphtheria toxoid conjugate vaccine

DTm, containing a hexa-His affinity tag, was produced in *E. coli* BL21 and expressed from plasmid pET22b-DT-51E148K. The bacteria were grown in Luria–Bertani (LB) medium in a 5 l fermentor at 37°C for 2.5 h. The temperature was reduced to 28°C, isopropyl β -D-thiogalactopyranoside was added to 1 mM, and growth was continued for 3 h. Periplasmic proteins were extracted by first resuspending the pelleted cells in 0.4 culture volume of 20% sucrose, 1 mM EDTA, 30 mM Tris–HCl, pH 8.0. After 10 min at room temperature, the mixture was centrifuged and the pelleted cells were suspended in the same volume of ice-cold 5 mM MgSO_4 . After 10 min on ice, cells were pelleted by centrifugation, and the mutant DT was purified from the supernatant fluid by affinity chromatography on a Ni^{2+} -chelate column (Novagen). The protein was further purified by size exclusion chromatography on Sephacryl S-200 (Pharmacia Fine Chemicals). The purified product (ca. 12 mg of purified protein was obtained per liter of fermentor culture) was monomeric and 95% pure, as judged by SDS-PAGE. Endotoxin (105 EU/ml) was removed using Detoxi gel (Pierce). The final solution was determined by the limulus amebocyte lysate assay (Associates of Cape Cod) to contain 3.4 EU/ml.

DTm was covalently coupled to purified GBS type III CPS by reductive amination with methods described previously [11]. Briefly, aldehydes were formed on 35% of the total sialic acid residues of type III CPS by limited sodium meta-periodate oxidation as described [11]. Oxidized type III CPS (5.1 mg) was added to 500 μ l of a 10 mg/ml solution of DTm in 0.3 M sodium phosphate buffer, pH 7.2. AN amount of 29 mg of purified sodium cyanoborohydride (Matreya, Inc.,

Pleasant Gap, PA) was added to the mixture and the conjugation reaction proceeded for 24 h at 37°C with mixing. The high molecular weight conjugate was purified from uncoupled components with a Superose 6 column (Pharmacia Fine Chemicals) by gel filtration chromatography. Fractions corresponding to the void volume of the column were collected and pooled, and uncoupled aldehydes were reduced by the addition of 2.2 mg of sodium borohydride for 1 h. The type III CPS-DTm conjugate (III-DTm) was dialyzed against water and dried by lyophilization. Carbohydrate analysis with purified type III CPS as the standard was performed with the phenol-sulfuric acid assay [12], and protein analysis with bovine serum albumin as the standard was performed with the BCA micro protein assay (Pierce Chemical Co., Rockford, IL).

2.2. Preparation of GBS type III CPS-rdHBcAg conjugate vaccine

The core gene (corresponds to bases 2647-411 of the duck hepatitis B virus genome, Genbank accession no. N60677) was cloned into the vector pKK223-3 and transformed into *E. coli* TB1 cells for expression. Typically, 24 l of culture was grown in medium containing 10 g yeast extract, 10 g tryptone, 5 g NaCl, 24 g glucose, and 50 µg/ml ampicillin. The bacteria were grown for 24 h at 37°C and then harvested. The rdHBcAg was produced as a soluble intracellular protein and was purified as described previously [13]. Typically, 0.5–1.0 g of purified protein is obtained from a 24 l culture. The endotoxin concentrations (Endotoxate®, Sigma-Aldrich Co., St. Louis, MO) in a typical preparation of rdHBcAg ranged between 0.16 and 1.6 ng/mg. The protein consists of 262 amino acids, and when expressed in *E. coli* (or any other host), the protein self-assembles into an essentially uniform population of approximately 26 nm particles (240 monomers per particle), held together by non-covalent interactions; it incorporates host RNA into its particulate structure, as does HBcAg [13].

Because of the particulate nature of the rdHBcAg, monitoring the coupling reaction using gel filtration chromatography in the absence of sodium dodecyl sulfate proved difficult in pilot experiments. Thus, SDS-PAGE was used to monitor the coupling reaction between oxidized type III CPS and rdHBcAg. To obviate the need to separate uncoupled from coupled

reactants, a series of small reactions were prepared to determine the concentrations that result in saturation of the added protein. To four microfuge tubes each containing 3 mg of oxidized type III CPS (54% oxidation of total number of sialic acid residues) was added 1, 2, 3, or 4.5 mg of rdHBcAg in a total volume of 0.5 ml; 3 μ l was removed from each reaction and stored at -80°C , sodium cyanoborohydride (15–24 mg) was added to each tube, and the mixtures were incubated at 37°C for 7 days with end-over-end mixing. Time 0 (before addition of sodium cyanoborohydride) and day 7 samples were appropriately diluted to achieve a concentration of 0.6 mg/ml and boiled for 5 min in 3 \times sample buffer [14]; 10 μ l of each mixture was separated on a 10% polyacrylamide minigel containing a 4% stacking gel (Genomics solutions, Ann Arbor, MI). The proteins were visualized by Coomassie blue staining. Since all conditions resulted in complete coupling of the rdHBcAg, all four reactions were pooled and dialyzed exhaustively against PBS at 4°C (molecular weight cut-off of dialysis tubing was 12,000–14,000). The coupled type III CPS–rdHBcAg (III–rdHBcAg), which was a white, cloudy particulate, was recovered from dialysis and stored at 4°C in PBS containing 0.01% thimerosal as a preservative. Carbohydrate analysis with purified type III CPS as the standard was performed with the phenol–sulfuric acid assay [12], and protein analysis with rdHBcAg as the standard was performed with the protein assay of Larson et al. [15].

2.3. Efficacy of GBS conjugate vaccines in mice

The efficacy of III–DTm and III–rdHBcAg vaccines was evaluated with the mouse maternal vaccination–neonatal challenge model of GBS disease as described [11]. Outbred CD-1 female mice (6–8-week-old) were vaccinated intraperitoneally with 2 μ g of type III CPS conjugated (III–DTm or III–rdHBcAg) or unconjugated (III+DTm or III+rdHBcAg), or saline mixed 1:1 with aluminum hydroxide gel (Alhydrogel 1.3%; Superfos Biosector, Vedbaek, Denmark) in a total volume of 0.5 ml, as described previously for GBS II-TT vaccine [16]. The unconjugated mixtures were prepared according to the compositional analysis of the conjugate vaccines; thus, the doses of CPS and protein delivered were similar to those

present in the conjugate preparations. A booster dose of vaccine was delivered by the same route approximately 3 weeks later. Dams were bred after they received the booster dose of vaccine, and their pups were challenged with an ordinarily lethal dose of GBS type III strain M781 within 48 h of birth. The challenge dose delivered in 0.05 ml ranged from 8.8×10^5 to 1.5×10^6 CFU per pup or 16,000–27,000-fold greater than the LD₅₀ for neonatal pups born to naïve dams. Survival was assessed 48 h after challenge. Serum was collected from dams before vaccination, before receipt of the booster dose, and following delivery.

2.4. ELISAs

The amount of GBS type III CPS-specific IgG in mouse serum was determined with use of a quantitative ELISA as previously described in detail [17].

DTm-specific IgG serum titers were determined by an ELISA using microtiter plates coated with 100 ng per well of DTm. Individual serum samples from dams were tested beginning at a 1:100 dilution and serially diluted three-fold across the plate. Goat anti-mouse IgG conjugated to alkaline phosphatase (Cappel, Organon Teknika Corp., Durham, NC) diluted 1:1000 was used as the secondary antibody. After 1 h of incubation, 100 μ l of Sigma 104 phosphatase substrate (1 mg/ml, Sigma–Aldrich Co., St. Louis, MO) was added to each well and the plate was developed for 30 min. Titers are the reciprocal serum dilutions that resulted in an absorbance of ≥ 0.3 at 405 nm.

IgG specific to the rdHBcAg in mouse serum was determined by an ELISA using microtiter plates coated with 100 ng of rdHBcAg per well. Serum samples were tested at a starting dilution of 1:100 and serially diluted two-fold across the plate. The plates were developed and titers determined as described above.

2.5. Opsonophagocytosis assay

The ability of vaccine-induced serum to opsonize GBS type III strain M781 for killing by human peripheral white blood cells *in vitro* in the presence of

human complement was determined by methods described elsewhere [18]. Pooled serum collected from dams after delivery was tested at a final assay dilution of 1:500. Results are the \log_{10} reduction in GBS CFU after 1 h of incubation.

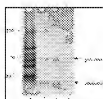
2.6. Statistical analysis

Significance of pup survival was determined with Fisher's exact test, and differences in antibody levels was determined with a two-tailed unpaired *t*-test (Instat, Graphpad Software, Inc.).

3. Results

3.1. III-rdHBcAg vaccine

Monitoring the conjugation of type III CPS to rdHBcAg by gel filtration chromatography proved difficult due to the particulate nature of the protein. However, when placed in SDS-containing sample buffer for PAGE, the protein disaggregated and monomers of approximately 30,000 Da and polymers of approximately 70,000 Da were visualized (Fig. 1, lane 2). Thus, SDS-PAGE was used to monitor the conjugation as polymers of large molecular weight would not enter the 10% acrylamide gel, and uncoupled protein could be separated and visualized. Increasing amounts of rdHBcAg were added to 3 mg of oxidized type III CPS in an effort to reach a protein concentration that would saturate the aldehyde binding sites in the CPS. Complete conjugation — as evidenced by absence of the protein bands — was achieved at a CPS:protein ratio of 3:1 after the addition of sodium cyanoborohydride (Fig. 1, lane 4). Because complete conjugation was also achieved at CPS:protein ratios of 3:2, 3:3, and 3:4.5, all four vials were combined, and the resulting III-rdHBcAg particulate vaccine was determined to be composed of 3.2 mg of CPS per ml and 2.5 mg of protein per ml.



Full-size image (10K)

Fig. 1. SDS-PAGE of GBS type III-rdHBcAg conjugate vaccine. Lane 1 shows the migration of molecular weight (in kDa) markers. Lane 2 shows the banding pattern (monomer and dimers) of 3 μ g of uncoupled rdHBcAg. Banding patterns of the reaction mixture containing 3 mg of oxidized GBS type III CPS and 1 mg of rdHBcAg before (lane 3) and 1 week after addition of sodium cyanoborohydride (lane 4). The absence of proteins in lane 4 indicates the formation of a high molecular weight polymer not able to enter the 10% polyacrylamide gel. Lanes 3 and 4 each contained 0.6 μ g of protein.

3.2. Immunogenicity and efficacy of III-rdHBcAg in mice

The type III CPS-specific geometric mean IgG concentrations (GMCs) in serum from III-rdHBcAg vaccinated mice before, 26 days after, and 53–62 days after the primary dose were 0, 2.3, and 22.3 μ g/ml, respectively. All of six mice vaccinated with III-rdHBcAg vaccine had between 3 and 160 μ g/ml of GBS type III CPS-specific IgG after delivery (53–62 days after receiving the primary dose of vaccine), whereas no CPS-specific antibody was elicited in mice that received an uncoupled mixture of type III CPS and rdHBcAg or saline (Table 1). Pup survival rates matched maternal type III CPS-specific IgG levels. Virtually all (overall survival rate of 97%) pups born to III-rdHBcAg-vaccinated dams survived an ordinarily lethal challenge with viable GBS type III strain M781 cells, whereas none of the pups born to dams that received either a mixture of type III CPS and rdHBcAg, or saline survived the bacterial challenge (Table 1).

Table 1. In vivo efficacy of group B *Streptococcus* type III conjugate vaccines and in vitro opsonophagocytic activity of vaccine-induced antiseruma

Vaccine	Dam	Dam type III CPS Ab ($\mu\text{g/ml}$)
III-rdHBcAg	1	54.2
	2	160.1
	3	17.9
	4	5.2
	5	49.0
	6	3.1
III + rdHBcAg	1	0.0
	2	0.0
	3	0.0
III-DTm	1	47.2
	2	26.2
	3	37.6
	4	95.5
	5	12.8
	6	102.4
III + DTm	1	0.0
	2	0.1
	3	0.1
	4	0.0
	5	0.0
	6	0.0
Saline	1	0.0
	2	0.0

Full-size table (<1K)

3.3. III-DTm vaccine

The purified III-DTm vaccine was composed of 82% (w/w) protein and 23% (w/w) carbohydrate (105% recovery).

3.4. Immunogenicity and efficacy in mice of III-DTm vaccine

The type III CPS-specific GMC in serum from III-DTm vaccinated mice before, 26 days after, and 53–62 days after the primary dose were 0, 5.0, and 42.4 $\mu\text{g/ml}$, respectively. All of six mice vaccinated with III-DTm had between 12 and 102 $\mu\text{g/ml}$ of type III CPS-specific IgG (Table 1), whereas all six dams that received the III CPS and DTm mixture had little (≤ 0.1 $\mu\text{g/ml}$) to no CPS-specific IgG ($P=0.005$). In all, 92% or greater protection was afforded to pups born to III-DTm-vaccinated dams following bacterial

challenge, whereas no protection was observed among the six litters born to dams that received the unconjugated mixture ($P < 0.0001$, Table 1).

3.5. In vitro functional activity of mouse antiserum

Pooled serum from dams that received the conjugated vaccines promoted in vitro opsonization that resulted in the reduction of GBS CFU by $>1.0 \log_{10}$ whereas serum from mice that received saline or uncoupled mixtures of type III CPS and protein was essentially non-opsonic (Table 1).

3.6. Immune response in mice to coupled and uncoupled carrier proteins

The geometric mean titer (GMT) of IgG specific for DTm rose following the primary vaccination with both the conjugated and unconjugated vaccines (Table 2). Increased titers of DT-specific IgG measured in serum obtained 4 to 5 weeks after administration of the booster dose among these two groups were suggestive of an anamnestic response to the secondary vaccination.

Table 2. Geometric mean titer of DTm-specific IgG in serum from mice vaccinated with type III CPS coupled to DTm (III-DTm), an uncoupled mixture of type III CPS and DTm (III + DTm), or saline

Vaccine	n	GMT (range) of DTm-spe	
		0	26
III-DTm	6	100 (100)	9727 (2700-2
III + DTm	6	100 (100)	2167 (300-
Saline	3	100 (100)	144 (100



Full-size table (<1K)

As seen with vaccines containing DTm, the GMT of rdHbcAg-specific IgG in serum from mice vaccinated with rdHbcAg-containing constructs rose following vaccination (Table 3). It is interesting to note that the carrier-specific GMT was greater in mice that received the coupled than the uncoupled protein.

Table 3. Geometric mean titer of rdHBcAg-specific IgG in serum from mice vaccinated with type III CPS coupled to rdHBcAg (III-rdHBcAg), an uncoupled mixture of type III CPS and rdHBcAg (III+rdHBcAg), or saline

Vaccine	n	GMT (range) of rdHB	
		0	26
III-rdHBcAg	6	100 (100)	317 (100)
III + rdHBcAg	6	100 (100)	10
Saline	3	100 (100)	635 ^a (100)



Full-size table (6K)



4. Discussion

The increased use of conjugation technology to generate immunogenic vaccines against encapsulated pathogens has resulted in the common use of some of the most successful carrier proteins namely, TT and CRM₁₉₇. Licensed conjugate vaccines *Haemophilus influenzae* type b use diphtheria or tetanus toxoid, CRM₁₉₇, or outer membrane proteins of *Neisseria meningitidis* group B [19] as carrier proteins and all seven CPSs in the newly approved heptavalent vaccine against *Streptococcus pneumoniae* are coupled to CRM₁₉₇ [20]. However, toxins from other bacteria have shown promise in animals to function simultaneously as carrier proteins and as immunogens. For example, murine antibody elicited by bacterial CPSs coupled to exotoxin A from *Pseudomonas aeruginosa*, or to a nontoxic peptide from *Clostridium difficile* toxin A, are functionally active against the carbohydrates and have toxin-neutralizing activity [21 and 22].

In this study, we demonstrated the use of a particulate recombinant viral protein rdHBcAg and a recently derived mutant protein of DT as suitable carrier proteins for GBS type III CPS. We chose to explore the ability of rdHBcAg to act as a carrier protein because hepatitis B viral core antigen can serve as an effective carrier for foreign epitopes that have been genetically engineered into the protein sequence at several selected sites [23, 24, 25 and 26]. In addition, animals inoculated with a single dose of HBcAg, without adjuvant, produce specific antibodies with titers normally greater than 1×10^6 .

which demonstrates that this protein is highly immunogenic.

Our early failures in monitoring the coupling of CPS to rdHBcAg by size exclusion chromatography (due to the aggregation of the protein in the SDS-free column buffer) were overcome by use of SDS-PAGE as a means of determining the degree of conjugation. The use of SDS-PAGE to monitor conjugation reactions was developed previously in our laboratory for the conjugation of type III CPS to alpha C-protein [2]. The use of SDS-PAGE and the "saturative coupling" approach resulted in the first GBS CPS conjugate vaccine that was particulate in nature and that proved to be immunogenic in mice, eliciting specific IgG that was functionally active both in vitro and in vivo.

The soluble DTm protein was easier to manipulate in the conjugation reaction than was rdHBcAg. However, it was similar to the rdHBcAg in its ability to provide T cell help to the conjugated type III CPS, as the III-DTm induced CPS-specific IgG with both in vitro opsonizing activity and the ability to protect against GBS disease in vivo. The usefulness of DT as a carrier protein for bacterial CPSs has been demonstrated in both animals and humans [27, 28 and 29]. Nevertheless, the newly developed DTm that has no enzymatic activity was also capable of acting as a carrier protein for GBS type III CPS.

Without doubt, a highly effective vaccine against GBS for use in the United States will contain the type-specific CPSs Ia, Ib, II, III, and V. Unless conjugation techniques such as reducing end linking of oligosaccharides [30] can reproducibly conjoin multiple CPSs onto a single protein carrier, a GBS multivalent vaccine, like the heptavalent pneumococcal conjugate vaccine [20], will be a combination of individual conjugates formulated as a single vaccine. Carriers other than TT or CRM₁₉₇ need to be developed and evaluated as potential alternatives. The preclinical data presented herein provide the rationale necessary to continue evaluation of rdHBcAg and the new DTm as potential carrier proteins for use in humans.

Acknowledgements

We thank Barbara Reinap, Kenneth Johnson, and

Jessica Bradford for expert technical assistance. We also acknowledge the many fruitful discussions with Dr. Dennis L. Kasper. This work was supported by NIH-NIAID contract AI-75326 and grant AI-22021 (RJC).

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